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Developing an Experimental System for Studying Early Events in Speciation in Drosophila

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Developing an Experimental System for Studying Early Events in Speciation in *Drosophila*

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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ABSTRACT

Speciation occurs when two populations of a species can no longer reproduce, either because of (1) pre-mating reproductive isolation, for example, populations having non-overlapping habitats, or evolving different courtship behaviors, or (2) post-mating pre-zygotic (PMPZ) isolation, where mechanical (*e.g.*, incompatible differences in genitalia), or gametic (*e.g.*, sperm and egg become incompatible) differences renders the strains unable to produce offspring. Previous work has suggested that when incipient species begin to experience reproductive isolation, a phenomenon called "reinforcement" can accelerate the process, by the rapid evolution of new pre-mating or PMPZ barriers. While the occurrence of reinforcement has been studied for many years, not much is known about how rapidly natural selection can create new reproductive barriers, or what the actual mechanisms are that are likely to arise [*e.g.*, pre-mating mechanisms like changes in courtship behavior, or PMPZ mechanisms like differential sperm storage or use by the female (cryptic female choice)]. My project focuses on using genetic engineering to create populations of genetically incompatible strains of *Drosophila melanogaster*, which then will be studied to understand the exact mechanisms whereby such strains might rapidly diverge from each other due to reinforcement. My research has used recombinant DNA methods to create two complex synthetic alleles (*A* and *B*), each consisting of four components. (1) The *Prot-B RFP* or *Prot-B GFP* sequences that encode sperm specific proteins tagged with red fluorescent proteins (RFP) or green

fluorescent protein (GFP) for the clear distinction of *A* and *B* sperm within a female's seminal receptacle; (2) the *3xP3 RFP* and *3xP3 GFP* cassettes that result in eye-specific expression of RFP and GFP to allow easy identification of which allele each fly carries; (3) the *attB* sequence that allows these constructs to be inserted into a specific chromosomal site, using the phiC31 integrase system for site-specific transformation; and (4) one of the two components of the GAL4/UAS system for targeted gene expression. The *A* allele carries the yeast transcriptional activator protein gene, *GAL4*, under the control of a constitutive promoter (i.e., the *Pros25* proteasome gene promoter). The *B* allele carries the dominant lethal gene *UAS-Poly-Q108* that is activated by GAL4. Thus, while either allele by itself is harmless, when both are present, such as in *AB* hybrids, the activation of *UAS-PolyQ108* leads to 100% pupal lethality.

These newly created incompatible *A* and *B* populations will be used for long-term experimental evolution studies. In these experiments, flies from both strains will be maintained together in population cages where they can freely mate and reproduce for many generations. Because the only productive matings will be between *A x A* and *B x B*, due to the hybrid incompatibility, there will be selective pressure to quickly evolve additional reproductive isolation mechanisms, which will then be identified and analysed in detail. If successful, this will confirm the concept of reinforcement, and furthermore give us clues about specific reproductive isolation mechanisms that are likely to quickly evolve.

INTRODUCTION

BACKGROUND

The formation of new species (speciation) is a very important, yet poorly understood, evolutionary process. Speciation occurs when two populations of a species can no longer interbreed, either because of (1) pre-mating reproductive isolation, for example, populations having non-overlapping habitats, or evolving different courtship behaviors, or (2) post-mating pre-zygotic (PMPZ) isolation, where mechanical (*e.g.*, incompatible differences in genitalia), or gametic (*e.g.*, sperm and egg become incompatible) differences renders two strains unable to hybridize. To understand how speciation occurs it is important to decipher how barriers to reproduction arise between discrete populations, and how these barriers become more established and stable over time, ultimately leading to complete reproductive isolation. Previous work has suggested that when incipient species begin to experience reproductive incompatibility, a phenomenon called "reinforcement" can accelerate the process, by the rapid evolution of new pre-mating or PMPZ barriers (Matute, 2010a). Reinforcement can thereby accelerate reproductive isolation between closely related subspecies and facilitate the completion of the speciation process.

Previous research by Daniel Matute (University of Chicago) has demonstrated reinforcement of PMPZ isolation by the rapid evolution of new reproductive barriers in crosses between two distinct, but closely related,

Drosophila strains, *D. yakuba* and *D. santomea*, that have recently diverged. *D. yakuba* females were shown to have developed an increase in gametic isolation when given an opportunity to mate with *D. santomea* males (Matute, 2009, 2010b).

One type of PMPZ mechanism that can arise involves the phenomenon of sperm precedence, which is defined as a non-random chance of fertilization when sperm of two males are present together within a twice-mated female. This can be seen in cases where a female mates with more than one male, and the stored sperm can therefore compete for fertilization of eggs (sperm competition). There are multiple possible mechanisms underlying sperm precedence – physical displacement and incapacitation of resident sperm by second-male sperm, female ejection of sperm, and biased use of competing sperm for fertilization. Mating with different males causes sperm competition. Mating with two males from the same line depends largely on the order of copulation and results in about 80% of the progeny being sired by the second of two males (Manier, et al., 2010c). When a female remates, the resident sperm can be physically displaced by the second-male sperm. Lüpold et al. (2011) showed that in competing sperm from two different lines the longer and slower sperm was displaced less easily than faster and shorter sperm, indicating consequences of pre-zygotic genetic variation of sperm. However, it was also shown that some of the resident sperm is first displaced without the presence of the second-male sperm, indicating a form of female preference, where the female controls and favors paternity of one male's sperm over the

other on the basis of selecting a particular trait (a phenomenon known as "cryptic female choice"). These are examples of possible mechanisms that could potentially evolve during the process of reinforcement.

While the occurrence of reinforcement has been studied for several years, it's occurrence is controversial and not much is known about how rapidly sexual selection can create new reproductive barriers, or what the actual mechanisms are that are likely to arise [*e.g.*, pre-mating mechanisms like changes in courtship behavior, or PMPZ mechanisms like differential sperm storage or use by the female (cryptic female choice)].

Here, I describe a novel, synthetic biology approach to create two populations of *D. melanogaster* that are engineered to be genetically incompatible. That is, while each strain is normally viable, and can breed with mates of their own strain with no fitness effects, hybrid matings are completely non-productive (*i. e.*, all of the offspring die in the pupal stage). These populations are designed to be easily distinguishable by virtue of having different fluorescent eye color phenotypes, and their sperm are fluorescently tagged to allow detailed observations of the stored sperm following mating, in order to identify and study aspects of PMPZ reproductive isolation mechanisms. While it is beyond the scope of my project, these populations will be used for long-term laboratory evolution studies in which evidence of reinforcement will be looked for, and detailed analysis of rapidly evolved reproductive isolation mechanisms will carried out.

STRATEGY FOR CREATING GENETIC INCOMPAIBILITY

To create the genetically incompatible populations of *D. melanogaster* I used recombinant DNA methods to create two complex synthetic alleles (called *A* and *B*), each consisting of four components. (1) The *Prot-B RFP* or *Prot-B GFP* sequences that encode sperm specific proteins tagged with red fluorescent proteins (RFP) or green fluorescent protein (GFP) for the clear distinction of *A* and *B* sperm within a female's seminal receptacle (Manier, et al., 2010); (2) the *3xP3 RFP* and *3xP3 GFP* cassettes that result in eye-specific expression of RFP and GFP to allow easy identification of which allele each fly carries (Horn, et al., 2000); (3) the *attB* sequence that allows these constructs to be inserted into a specific chromosomal site, using the phiC31 integrase system for site-specific transformation (Venken, et al., 2006) ; and (4) one of the two components of the GAL4/UAS system for targeted gene expression (Brand and Perrimon, 1993). The *A* allele carries the yeast transcriptional activator protein gene, *GAL4*, under the control of a constitutive promoter (i.e., the *Pros25* proteasome gene promoter). The *B* allele carries the dominant lethal gene *UAS-Poly-Q108* that is activated by GAL4. Thus, while either allele by itself is harmless, when both are present, such as in *AB* hybrids, the activation of *UAS-PolyQ108* leads to 100% pupal lethality.

ProtB-GFP* and *ProtB-RFP

To tag the sperm heads with fluorescent markers so that the sperm from different males could be discriminated, and details of their motility, storage, and use for fertilization in twice-mated females, I included either the *ProtB-GFP* or *ProtB-RFP* cassettes in my *A* and *B* constructs. These sequences have the *D. melanogaster* protamine gene, *ProtB*, tagged with the coding sequence of Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP). Protamines are sperm-specific chromosomal proteins that are abundantly expressed in the sperm head, enabling easy visualization of stored sperm within a dissected female reproductive tract (Manier, et al., 2010).

3xP3-GFP* and *3xP3-RFP

3xP3 is an eye-specific promoter sequence that binds the highly conserved Pax6 transcription factor to activate transcription of the adjacent gene. Horn, et al. (2000) engineered DNA cassettes that place either GFP or RFP downstream of this promoter to create a genetic marker that is easily scored in the adult fly (as either green or red fluorescent eye spots).

FC31 site-specific recombinase system for targeted insertion

A common method for introducing cloned plasmid constructs into the Drosophila genome is using the P-element mediated germline transformation (Rubin and Spradling, 1982). However, as this conventional transformation method is somewhat inefficient and the randomness of construct insertion

might affect the transgene expression, we used the site-specific recombinase, ϕC31 that mediates crossovers between a bacterial attachment site (*attB*), included as part of our engineered A and B constructs, and a phage attachment site (*attP*), that has been inserted as a transgene into the *D. melanogaster* genome (Venken, et al., 2006). This crossover event, which is quite efficient, produces two new sequences, *attR* and *attL*. Thus inserted constructs are not recognized by the integrase and cannot be further modified or excised from the sequence (Bateman et al., 2006). The ϕC31 integrase used for these experiments was germ-line-specific and came from a transgenic source, eliminating the need for an mRNA helper plasmid (Bischof et al., 2006). Figure 1 shows a schematic representation of the plasmid integration.

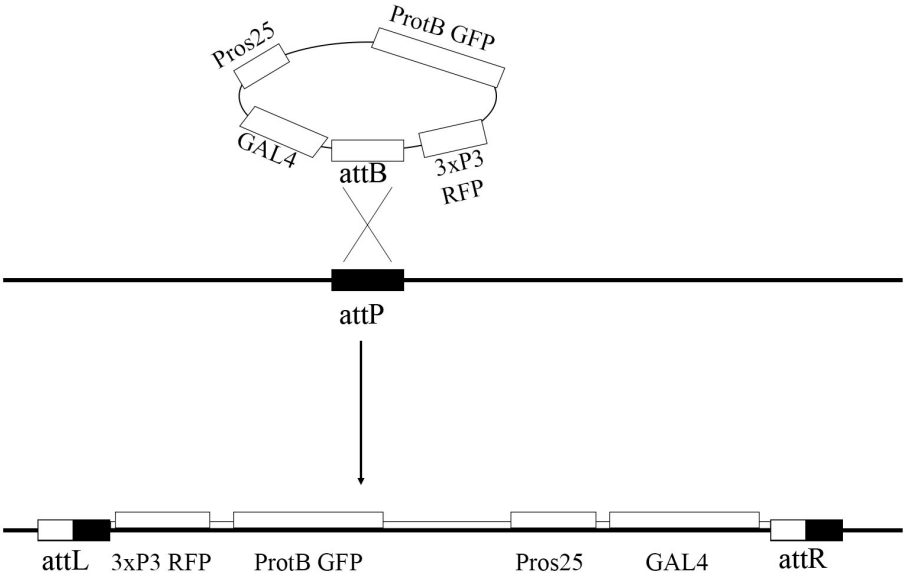


Figure 1 Mechanism of site-specific plasmid integration. A crossover between the bacterial attachment site, attB, and the phage attachment site, attP, causes a creation of two new sites, attL and attR, and incorporation of the entire vector into the genome.

UAS/GAL4 system for targeting lethality of hybrids

To introduce specific, ectopic expression of the transformed plasmid construct, and selectively activate a gene throughout the developing fly, the UAS/GAL4 binary system was used (Brand and Perrimon, 1993). UAS (the Upstream Activating Sequence), is the binding site for the yeast transcriptional activator protein GAL4. When GAL4 binds to UAS, an adjacent promoter will be activated, leading to high levels of transcription of that UAS-gene construct (Figure 2). In this experiment I made two different constructs, one containing the *UAS-rpr.c* sequence (Aplin and Kaufman, 1987) and the other having *UAS-PolyQ108* (Marsh, et al., 2000). Both of these genes induce cell toxicity when expressed in the fly, and therefore can act as dominant lethal genes. I constructed these two constructs since it was not known if these synthetic genes would act as expected when paired with a particular GAL4 driver.

For the GAL4 component of the system, I synthesized a construct placing the *GAL4* coding sequence downstream of the constitutive *Pros25* promoter. Pros25 is a subunit of the proteasome, a multi-subunit complex that is responsible for programmed proteolysis in all eukaryotic cells (Neuburger, et al., 2006). It was anticipated that this construct would ubiquitously express GAL4 when introduced as a transgene. By itself this would have no adverse

effects on the developing fly. But if this construct was present in the same fly as one carrying either *UAS-rpr.c* or *UAS-PolyQ108*, it would trigger widespread expression of these toxic proteins and lead to death.

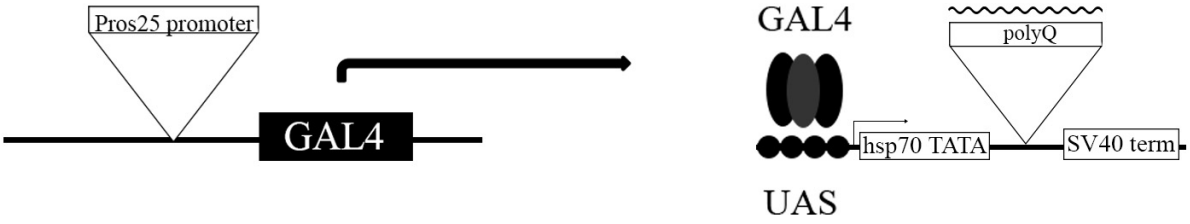


Figure 2 Mechanism of UAS-GAL system. Expression of GAL4, a yeast transcriptional activator, is driven by the *Pros25* promoter. Active GAL4 binds to the Upstream Activating Sequence (UAS) and guides the transcription of toxicity-inducing polyQ gene.

Buffer and solution ingredients

TE: 10 mM Tris, 1mM EDTA (pH 7)

Resuspension buffer: 50mM Tris-Cl, 10mM EDTA, 100μg/ml RNase A pH 8.0, stored at 4°C

Lysis buffer: 200mM NaOH, 1% SDS, stored at 25°C

Neutralization buffer: 3.0M potassium acetate, pH 5.5, stored at 25°C.

TAE buffer: 0.04M Tris-acetate, 0.002M EDTA, pH 8.0, stored at 25°C.

Agarose gel electrophoresis

Electrophoresis of DNA samples was usually done using 0.7% agarose (Fisher Scientific) in 150 ml TAE buffer with 2 μg/ml of ethidium bromide. ~2μl of bromophenol blue was added to each DNA sample before running. The gel was usually run for 35 min at 75 volts, with a DNA Hi-Lo ladder used to approximate DNA fragment size.

Isolation of plasmid DNA: Wizard prep

DNA isolation was performed using the Wizard® Plasmid Prep manual from Promega. A single *E. coli* colony was transferred to a 12 ml

sterile capped tube containing 1.5 ml Terrific Broth (TB) and 5μl of 20mg/ml ampicillin using a sterile pipette tip. Inoculations were then placed in a 37°C incubator overnight with shaking. Next, cells were transferred to a 1.5 ml microfuge tube and centrifuged at 5000 rpm for 3 min. Supernatant was decanted and disposed of, and the remaining pellet was dissolved in 200μl wizard cell resuspension solution. Next, 200μl of Wizard® lysis solution were added. After ~ 3 minutes, 200μl of neutralizing solution was added. Tube was then centrifuged at 10000 rpm for 15 minutes, at which point the precipitant formed a pellet. The supernatant was removed and filtered through a vacuum using Wizard® filter cartridges and syringe barrels containing DNA binding resin. Filters were then washed with wash solution, and briefly dried via centrifugation by placing filters on top of a 1.5 ml microfuge tube with removed caps. Subsequently, 75μl of TE buffer was added and allowed to elute the DNA for 5 minutes. The DNA containing solution was then removed from the filter and collected into a sterile microfuge tube via centrifugation (10000 rpm x 5 min).

Isolation of plasmid DNA: Midi Prep

DNA isolation for embryo microinjection was prepared using the QIAGEN® midi-prep kit. A starter culture was inoculated from a desired colony in 3ml of LB containing 15 μl ampicillin. The culture was incubated overnight at 37°C w/shaking. Next, the starter culture was dilutes 1/500 in 25

ml LB. It was then grown overnight at 37°C with vigorous shaking. The cells were subsequently harvested by centrifugation at 6000 rpm for 15 min at 4°C and resuspended in 2ml of buffer P1. Then, 2ml of buffer P2 were added, the mixture was shaken repeatedly, and incubated at room temperature for 3 min. Next, 2ml of Buffer S3 were added to the lysate, mixed by inverting, and placed in the barrel of the QIAfilter cartridge, were it was subsequently incubated at room temperature for 3 min. The lysate was then filtered into a new tube, where 2 ml of buffer BB were added. The lysate was mixed by inverting the tube and transferred to a tube attached to the CompactPrep column. The solution was then drawn through the column by using a vacuum. 0.7 ml of buffer PE was then added to the column to wash the DNA. It was then removed by applying the vacuum for 10 minutes.

The column containing the DNA was then placed in a clear 1.5 microcentrifuge tube and eluted by adding 100 μl of buffer EB to the column and centrifuged for 60s.

Agarose gel analysis was then performed to determine the yield and confirm identity of the DNA.

Isolation of plasmid DNA: Mini Prep

A single colony was inoculated in 1.5 ml of LB in a 12 ml sterile tube and incubated at 37°C with shaking overnight. Next, the cultured solution was poured into a 1.5 ml microfuge tube and centrifuged at 12000 rpm for 3 min.

Supernatant was poured off and the pellet resuspended in 200 µl of the resuspension buffer. Next, 200µl of the lysis buffer was added and the mixture was inoculated at 25°C for 3 minutes allow cells to lyse. Then, 200 µl of neutralization buffer were added and incubated on ice for ~ 8 min. Next, the mixture was centrifuged at 12000 rpm for ~ 8 min at 25°C. Supernatant was then transferred to a fresh tube, and 450µl of isopropanol were added and mixed. The resultant mixture was incubated on ice for 10 minutes and centrifuged at 12000 rpm for the next 10 min. Supernatant was then decanted and the pellet was washed with 70% ethanol. Pellets were then dried using a vacuum for 15 minutes. Next, 75 µl of TE was added to each pellet. Samples were then incubated at 37°C for 15 minutes to dissolve the DNA and degrade the RNA. Tubes were mixed by vortexing.

DNA primer synthesis

Primers required for PCR were ordered from the Sigma-Aldrich Company. Primers were designed in such a way to introduce sequences recognized by the restriction enzymes used in subsequent element ligation. The restriction enzymes were chosen on the basis of their uniqueness in the sequences. All samples were received dry and dissolved in TE to make a stock solution of 250µM. Primers used were as follows:

attB 3’ (3.attB8308)	ATTAAGGCCGGCGCGCCATCGATAAC
attB 5’ (5.FseattB7553)	AGGCCGGCCAGTTATTGGTGCCCTTAAACGCC
3xP3 EGFP 5’	AAGATCTAATTCGAGCTCGCCCGGGGATCTAATTC
3xP3 EGFP 3’	AAGATCTTGTACGCGTATCGATAAGCTTTAAG
3xP3 RFP 5’	ACTGCAGTATCGAATTTACTATAGTATCCCCG
3xP4 RFP 3’	ACTGCAGATTATGAGATCGAAAGGGTCTACGA
Pros25 5’	AAAGATCTGGCCGGCCCTTTAAAGTGTACCCACTG
Pros25 3’	AAGATCTGGCCGGCCCTTTAAAGTGTACCCACTG
GAL4 5’	GAGATCTAGGGTACGAACAAGCGCAGCTGAACAAGC
GAL4 3’	AGGCCGGCCGCTCTAGAACTAGTGGATCTAAACGAG
pUAST 5’	ACGGCCGGCCATACATACTAAGGCCTTCTAG
PUAST 3’	TTGGCCGGCCGGGCTGCATCTCTCCGGATCCAA
5.3pUAST polyQ 5’	TGGCCGGCCTGGTACTTCAAATACCCTTGG
3.2pUAST polyQ 3’	AGGCCGGCCGTGGGGTTTGAATTAACTCATAA
Polyub 5’	AAGATCTGGCCGGCCGGAACGCAGCGACAGGGATTCC
Polyub 3’	AAGATCTGGATTTTGGATTATTCTGCGGG
ArmBF 5’	GAGATCTGGCCGGCCAGCTGCTGTGACCATAA
ArmB 3’	GAGATCTACCACACCTGCAAGAAAGAGAC
αtubBF 5’	GAGATCTGGCCGGCCCAACTAGTCCTGCAA
αtubB 3’	GAGATCTTTCAGCTGTGGATGAGGAGGAAGG

Polymerase Chain Reaction (PCR)

A thin-walled 05.ml PCR tube was used for all reactions. 2µl of a 1/500 dilution of the plasmid DNA was added to 2.5µl of 10X GoTaq (Promega) reaction buffer, 2µl of dNTP mix (containing 200µM of each nucleotide), 1µl of each primer (using a 5µl diluted stock), 1µl of *Taq* or *Pfu* polymerase was added. The tubes were then mixed and centrifuged, and subsequently denatured at 95 °C for five minutes. Then, the tube was typically subjected to 35 cycles of following: 94 °C for 60s, 58 °C for 60s, and 72°C for 60s. The time of the elongation step was adjusted according to the length of the desired DNA (1 min per 1kb of DNA product). Annealing temperature was adjusted to the variations in the primers' melting temperature. At the end of the cycle, the mixture was incubated at 72 °C for 8 minutes and stored at 4 °C until needed. The samples were then stored at -20°C and 5µl of sample was used to confirm the concentration using gel electrophoresis.

TOPO cloning of PCR products

TOPO TA cloning kit (Invitrogen) was used to clone the PCR product into a usable vector using Topoisomerase I. TOPO plasmids used were either pCR 2.1-TOPO or pCR 4.1-TOPO (Figures 3 and 4, respectively). The reaction was performed using 1.5µl of fresh PCR product, 1 µl of diluted salt

solution, 1 μl of TOPO vector and 1.5 μl of H_2O for a total reaction volume of 5 μl. The reaction was then incubated at 25°C for 30 min.

Vector ligation

The fragments to be ligated were prepared by appropriate restriction digestion. Shrimp Alkaline Phosphatase was also added to remove the 5' phosphates to prevent reclosing the vector. Ligations were usually performed in a 500μl microfuge tube. Typically, 6μl of insert DNA was added to 3μl of plasmid vector DNA and combined with 1.5μl of 10X ligation buffer (provided with T4 ligase), 1.0 μl of T4 ligase and 3.5μl H_2O for a total volume of 15μl. Reactions were incubated at 14 °C overnight

DNA sequencing

Sequencing was performed by the GeneWiz Company using pre-defined sample sequencing. 10μl of ~ 50 ng/μl DNA and 5 μl of the primers provided by GeneWiz (either T7 or M13-Reverse) were used for sequencing.

Electrocompetent cell preparation

A colony of *E. coli* strain DH5α was inoculated in 5ml LB broth and grown overnight at 37°C w/shaking. The next day, 200 ml of LB was

inoculated with 3ml of the fresh overnight culture and shaken at 37°C for ~2.5 hrs. Cells were then centrifuged at 5000 rpm g at 4°C for 5 min and the resulting pellet was subsequently washed multiple times with ice cold 10% glycerol to remove salts interfering with the electroporation. ~70 µl aliquots of cells were quick frozen in a dry ice and ethanol bath and stored at -70°C until needed.

Transformation of *E. coli* by electroporation

1.5 µl of DNA was added to thawed electrocompetent cells. DNA was diluted 500x with the exception of ligation reactions. The mixture was then transferred into an electroporation cuvette, which was then submitted to a ~1850V shock. Immediately after the shock, the cells were transferred to a 300µl LB solution and incubated at 37°C for ~ 45 min w/shaking. Next, 50µl of 2% X-gal, 15µl of ampicillin solution (20 mg/ml), 10µl of IPTG, and 3 ml of melted top agarose were added and spread on the previously warmed up and labelled LB + amp plates. After solidifying (~ 7-10 min), the inverted plates were placed in the 37°C incubator overnight.

Restriction enzyme digestion

Digestion was usually performed using 1µl of the desired enzyme, 12 µl of H_2O, 2µL of 10X buffer (specific to each enzyme), and 5µl of DNA for

a total volume of 15 μl. For digestions with *Fse*I, proportions were adjusted to ensure maximum efficiency and 1.5 μl of the enzyme and 1.5 μl of DNA were used. Water volume was adjusted accordingly for the total volume of 20 μl. Tubes were inoculated at 37°C and the digestion time varied from 45 min – 1.5 hrs. Results were confirmed via gel electrophoresis.

Egg microinjection and generation of transgenic lines

The completed plasmid constructs were subsequently used for germline transformation. 15μg of plasmid of interest purified using the midi-prep method was precipitated with 1/10 volume 3M NaOAC and 2 volumes of ethanol. Next, the tube was centrifuged for 30 minutes at 13000 rpm. The pellet was then washed with ethanol and resuspended in 50μl injection buffer (5mM KCl, 01. mM NaPO4 buffer at pH 6.8). 4μl of phenol red (Sigma-Aldrich) was then added to 15μl of the DNA in the injection buffer and used for injections.

Eggs were collected from 4-6 day old adults of the strain carrying an X-linked transgenic source of ϕC31 integrase, driven by a germline specific promoter, and a Chromosome 3 linked *attB* site [Bloomington Stock Center stock #35569, genotype y^1 *w* P{*$y^{+t7.7}$ *= nos-phiC31/int.NLS}X; PBac{*y^+*-attP-9A}VK00027* (Venken, et al., 2006)]. Egg collection plates containing agar, apple juice, glacial acetic acid and mold inhibitor (10% methyl-p-hydroxybenzoate in ethanol) with added yeast were put on the top of the

bottle and the bottle was inverted. Flies were then placed overnight in a darkened area to adjust to the new setting, and thereafter were allowed 30-40 minutes for the fertilized eggs to be laid. Early stage embryos were collected and lined up on the edge of a piece of double sided tape placed on a cover slide, with each posterior end facing the same direction. The embryos were then placed in a Petri dish containing desiccant for 5 minutes. A glass injection needle was loaded with DNA using capillary action, and each embryo was subsequently injected at the posterior end (the future site of germ cells) to dispense the DNA. Injection was normally completed within 90 minutes from fertilization and before the syncytial blastoderm stage to ensure the incorporation of DNA into the pole cells. Injected eggs were incubated in a humid chamber for 1-2 days and hatched larvae transferred to a food ial to complete development. Upon eclosion, the fly was crossed to a mate from the wild-type *LHm* strain, and progeny scored for transformants based on the GFP or RFP eye spots (from the *3xP3-GFP/RFP* component of the injected construct). The transformants were backcrossed for several generations to *LHm* and then made homozygous to create a stable line.

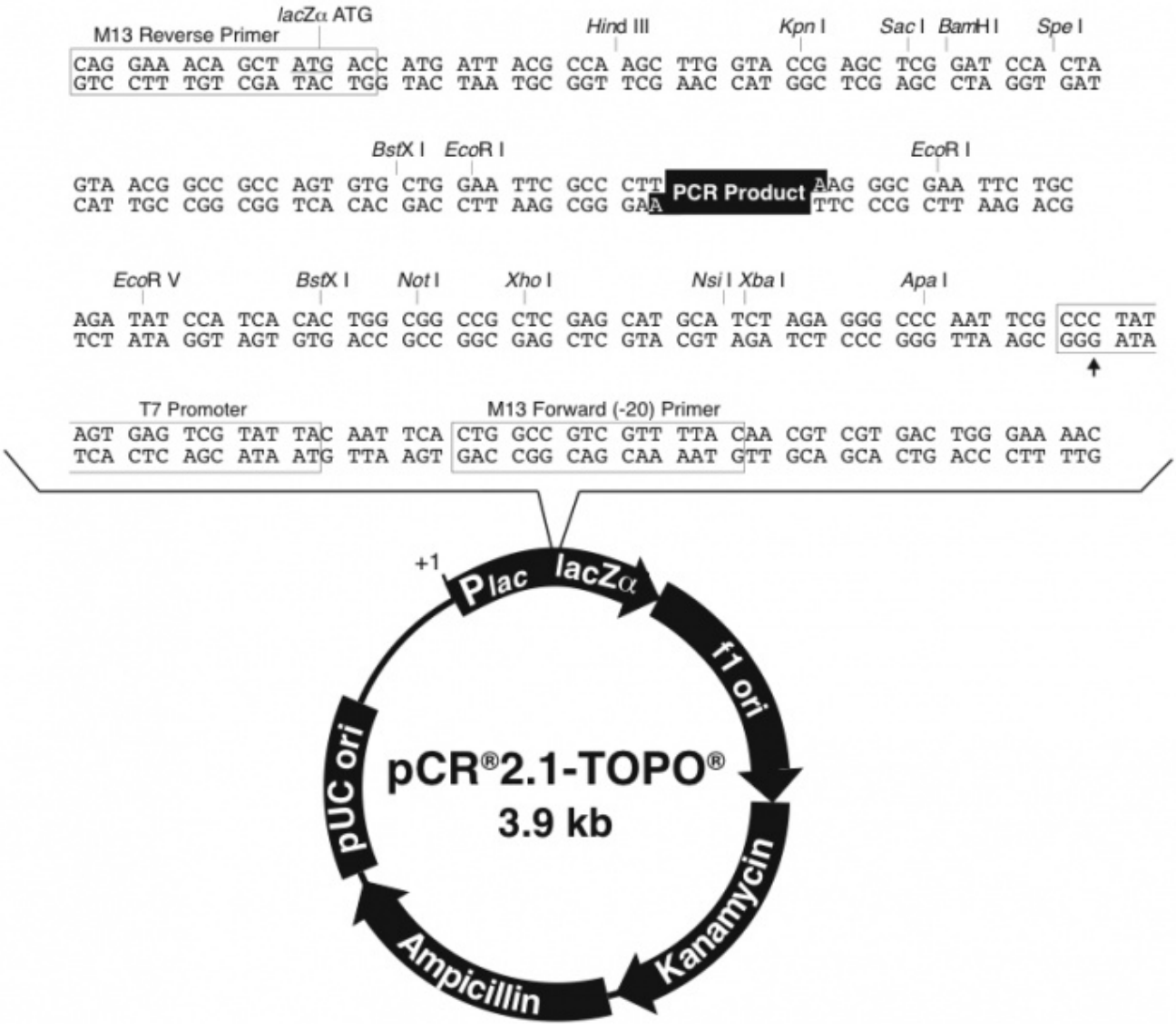


Figure 3. pCR 2.1-TOPO. Site of PCR product insertion. Invitrogen.com

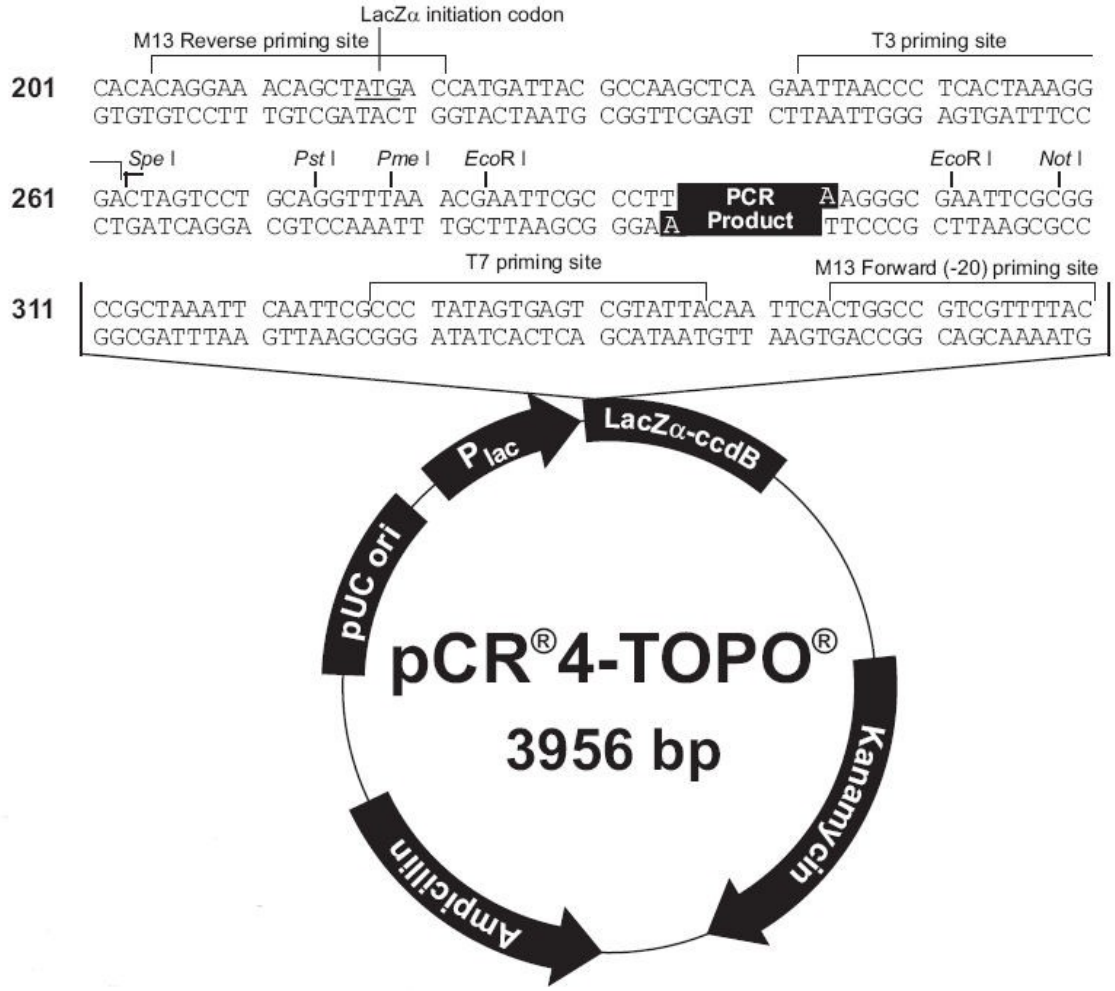


Figure 4 pCR 4-TOPO. Site of PCR product insertion. Invitrogen.com

Construction of flies containing the synthetic *A* allele

The synthetic *A* allele construct contains the ProtB-GFP, 3xP3-RFP, attB and Pros25-GAL4 components (*i. e.*, *Pros25-GAL4/attB/3xP3-RFP/ProtB-GFP*). Transgenic flies carrying this complex gene will have RFP eyes, GFP-tagged sperm and should ubiquitously express GAL4 throughout development. This complex locus will be integrated into the 3rd Chromosome at the site of the *attP* sequence carried by the host strain VK00027. Figure 5 shows the multi-step cloning strategy for the construction of this gene complex and generation of a stable transgenic line.

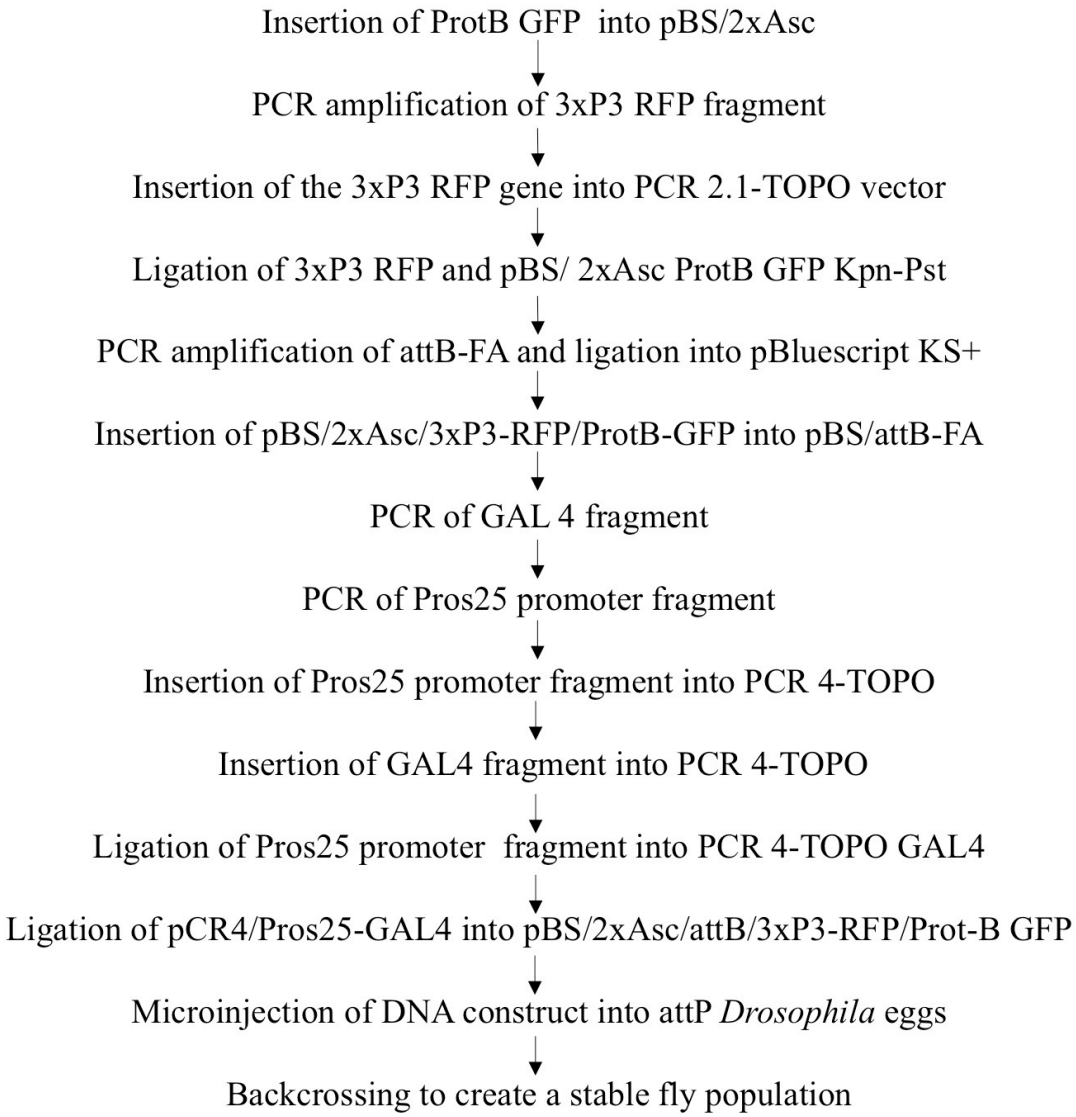


Figure 5. Multi-step cloning strategy for the construction of Pros25-GAL4/attB/3xP3 RFP/ProtB GFP

First, we created a plasmid combining the eye-specific 3xP3-RFP sequence with that encoding the ProtB-GFP fluorescent sperm protein. The 5.0 kb *KpnI-PstI* fragment of plasmid pBS/ProtB-GFP4 (Manier et al., 2010) containing the GFP-tagged *ProtamineB* gene (also called *Mst35Bb*) (Jayaramaiah Raja and Renkawitz-Pohl, 1993) was inserted into the *KpnI-PstI* sites of pBS/2xAsc, a modified pBluescript KS+ vector with two *AscI* sites flanking the multiple cloning sites to create pBS/2xAsc/ProtB-GFP (Figure 6A)

Next, a 1.4 kb fragment containing the DsRed coding sequence downstream of the eye specific promoter 3xP3 was subsequently PCR amplified from the plasmid *pBac{GMR03xP3-DsRed}* (provided by Max Scott, NC State University) using primers 5.3xP3DsRedPst and 3.3xP3DsRedPst (see p. 19 for this and all subsequent primer sequences) containing *Pst*I sites at their 5' ends. The product was cloned into pCR2.1 using the TOPO TA cloning system (Invitrogen). The 1.4 kb *Pst*I fragment was then excised and ligated into the unique *Pst*I site of pBS/2xAsc/ProtB-GFP to give pBS/2xAsc/3xP3-RFP/ProtB-GFP (Fig. 6b).

I chose to use contrasting colors of fluorescent proteins (that is, 3xP3-GFP and ProtB-RFP in one construct, and 3xP3-RFP and ProtB-RFP in parallel construct) in each construct to ensure their proper amplification and ligation during the multiple cloning steps. That is, if the GFP or RFP sequences were present twice in the same construct, there is a chance of intra-

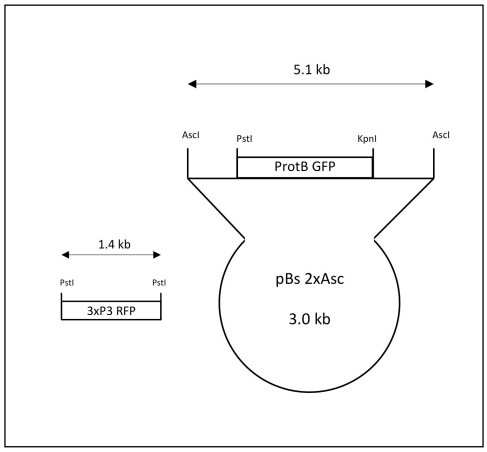
plasmid recombination, due to the repetitiveness of the sequences, and this could lead to deletion of sequence from the final amplified plasmid.

After creating the pBS/2xAsc/3xP3-RFP/ProtB-GFP plasmid, a 771 bp fragment containing the *attB* bacterial attachment site, a target of ϕC31 integrase, with an *Fse*I site at one end and an *Asc*I site at the other, was PCR-amplified from plasmid attB-P[acman]-ApR (Venken et al., 2006) using primers 3.attB8308 and 5.FseattB7553. The product was then cloned using the TOPO TA cloning system to give pCR4/attB-FA. The insert was then cut out with *Eco*RI and ligated into the *Eco*RI site of pBluescript KS+ to yield pBS/attB-FA. The 6.4 kb *Asc*I fragment of pBS/2xAsc/3xP3-RFP/ProtB-GFP was then ligated into the *Asc*I site of pBS/attB-FA to give pBS/attB/3xP3-RFP/ProtB-GFP (Fig. 6 c, d).

A 2.3 kb fragment containing the GAL4 coding region was PCR-amplified from plasmid pGATB (Brand and Perrimon, 1993) using primers GAL4 5' and GAL4 3' and TOPO-TA cloned into pCR4 to give pCR4/GAL4. The resulting plasmid contains a unique *Bgl*II site near the start of the *GAL4* coding region and an *Fse*I site downstream of the transcription termination sequence.

The upstream promoter region of the constitutively active *Pros25* gene (encoding the proteasome α2 subunit) was PCR-amplified from plasmid pW8/Pros25-2.0 KB (Neuburger et al., 2006) and the 0.9 kb product was first TOPO-TA cloned into PCR4, and then cut out with *Bgl*II and ligated into the *Bgl*II site of pCR4/GAL4 to give pCR4/Pros25-GAL4 (Fig. 7a). Because this

fragment could insert in either orientation, it was necessary to determine which clones had the proper insertion of the *Pros25* promoter that would drive transcription of the adjacent GAL4 coding region. Clones with the right orientation were identified by restriction enzyme digestions. Next, the *Pros25-GAL4* fragment was excised from pCR4/Pros25-GAL4 using the *Fse*I sites flanking the constructs and ligated into the unique *Fse*I site of pBS/2xAsc/attB/3xP3-RFP/Prot-B-GFP to create the final construct pBS/Pros25-GAL4/attB/3xP3-RFP/ProtB-GFP (Fig. 8). This construct will be referred to below as simply Pros25-GAL4-RG.



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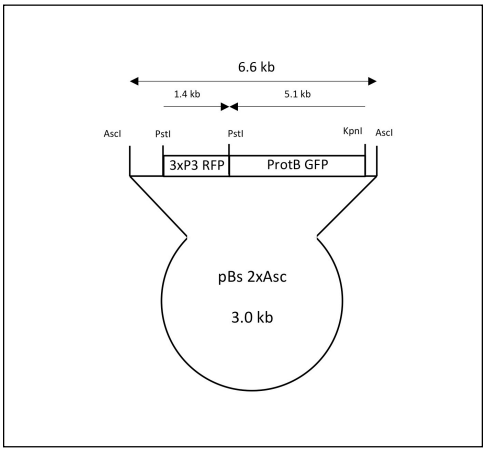

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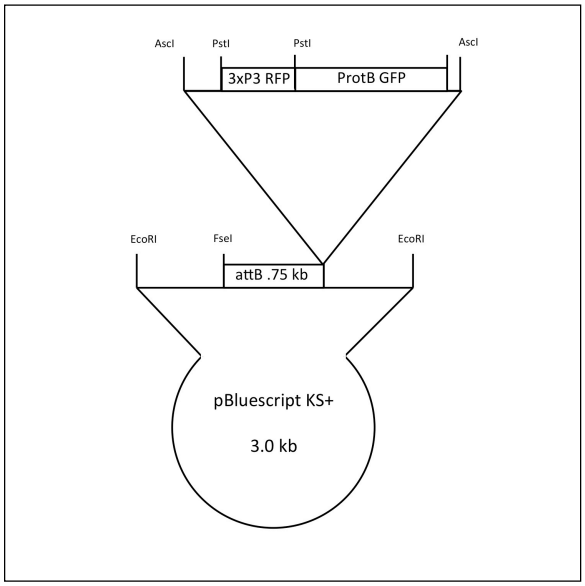

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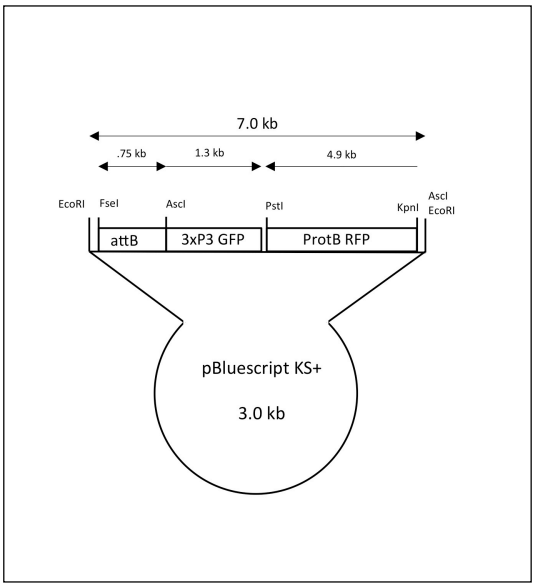


D

Figure 6 Construction of pBS/attB-FA/3xP3 RFP/ProtB-GFP. **A.** 3xP3 fragment in pCR-2.1 TOPO is ligated into pBS/2xAsc/ProtB GFP **B**. The obtained pBS/2xAsc/3xP3RFP/ProtB GFP construct
C. Insertion of 3xP3 RFP/ ProtB GFP AscI-AscI fragment into pBS/attB-FA. **D.** Final pBS/attB/3xP3 RFP/ProtB GFP construct

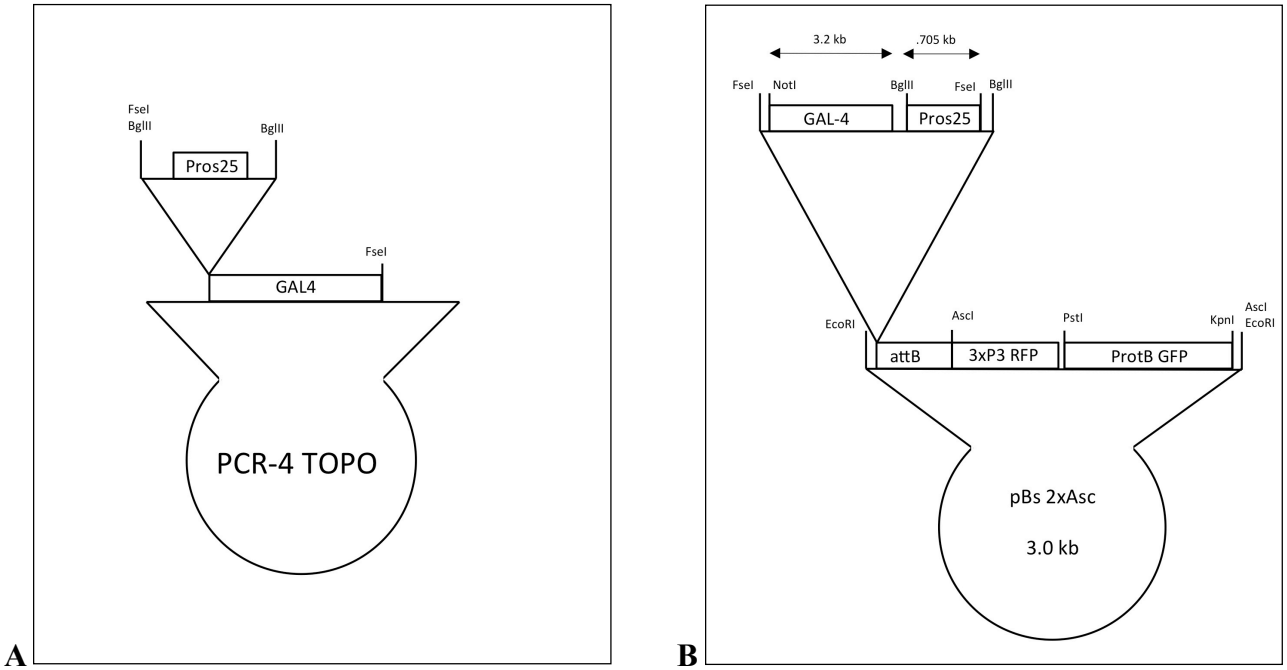


Figure 7 Construction of pBS/Pros25-GAL4/attB-FA/3xP3 GFP/ProtB-RFP. **A.** Insertion of Pros25 *FseI-BglII* fragment **B**. The obtained pBS/2xAsc/3xP3RFP/ProtB GFP construct
C. Insertion of 3xP3 RFP/ ProtB GFP AscI-AscI fragment into pBS/attB-FA. **D.** Final pBS/attB/3xP3 RFP/ProtB GFP construct

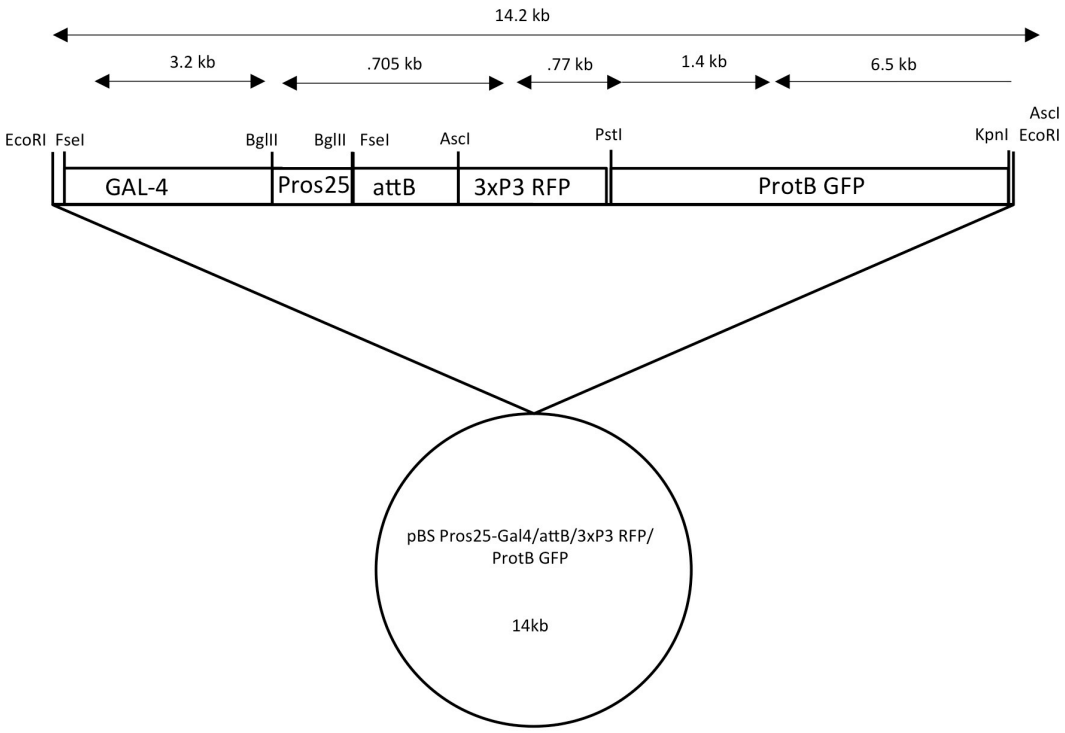


Figure 8 Construction of pBS/Pros25-GAL4/attB-FA/3xP3 GFP/ProtB-RFP. Complete construct in pBluescript KS+ vector.

Creation of flies containing the synthetic *B* allele

The synthetic *B* allele construct contains the ProtB-RFP, 3xP3-GFP, attB and either UAS-PolyQ108 or UAS-rpr.c components (*i. e.*, *UAS-PolyQ108/attB/3xP3-GFP/ProtB-RFP* or *UAS-rpr.c/attB/3xP3-GFP/ProtB-RFP*). Transgenic flies carrying this complex gene will have GFP eyes, RFP-tagged sperm and should respond to the presence of GAL4 by expressing a cytotoxic protein [either PolyQ108 (Marsh, et al., 2000) or the apoptotic protein reaper (Aplin and Kaufman, 1987)]. This complex locus will be integrated into the 3rd Chromosome at the site of the *attP* sequence carried by the host strain VK00027. Figure 9 shows the multi-step cloning strategy for the construction of this gene complex and generation of a stable transgenic line.

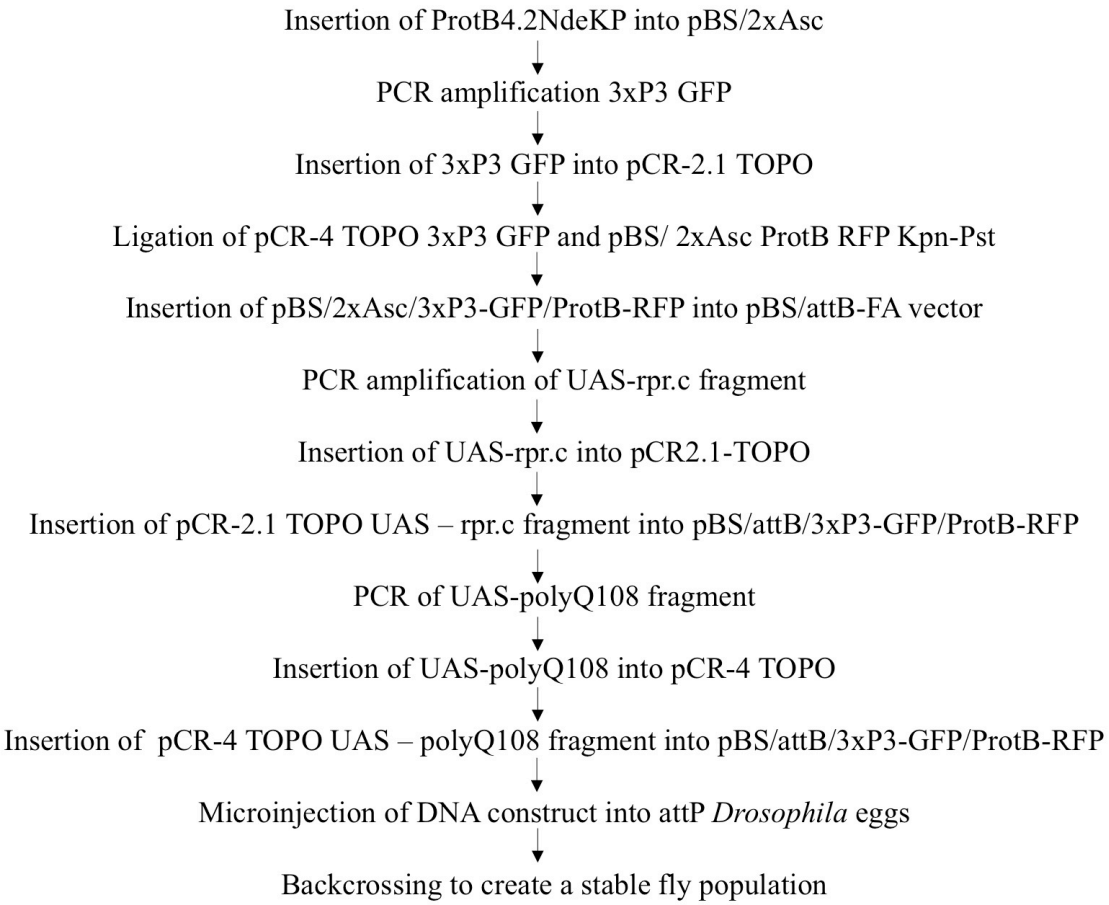


Figure 9 Multi-step cloning strategy for the construction of Pros25-GAL4/attB/3xP3 RFP/ProtB GFP

In the first step of this multi-step cloning strategy, the red fluorescent protein mCherry coding sequence, flanked by *Nde*I sites, was PCR-amplified from pmCherry (Invitrogen), and TOPO-TA cloned into pCR2.1 (Invitrogen) to give pCR2.1/CherryNde. Next, the 4.2 kb *Kpn*I-*Pst*I fragment of pBS/ProtB4.2NdeKP, containing the *ProtB* gene with an engineered *Nde*I site at the C-terminus of the coding region (Manier et al, 2010), was subcloned into the *Kpn*I-*Pst*I sites of pBS/2xAsc to give pBS/2xAsc/ProtB4.2NdeKP. The *Nde*I mCherry cassette was then cut out of pCR2.1/CherryNde and ligated into the unique *Nde*I site of pBS/2xAsc/ProtB4.2NdeKP to give pBS/2xAsc/ProtB-Cherry5.0KP.

A 1.3 kb fragment containing EGFP downstream of the eye-specific promoter 3xP3 was PCR amplified from pBac/3xP3-EGFPaf (Horn and Wimmer, 2000) using primers 3xP3 EGFP 5' and 3xP3 EGFP 3', and cloned into pCR2.1 using the TOPO-TA system to give pCR2.1/3xP3 BglI #5. The insert was then cut out with *Bgl*II and inserted into the unique *Bam*HI site of pBS/2xAsc/ProtB-Cherry5.0KP to give pBS/2xAsc/3xP3-GFP/ProtB-RFP (Fig. 10 a,b). In this construct, the use of *Bam*HI to cut the fragment out would be inefficient as there are other *Bam*HI sites within the sequence. Instead, *Bgl*II was used since it is an enzyme that leaves *Bam*HI compatible sticky ends, and there are no *Bgl*II sites within the pBS/2xAsc/ProtB-Cherry5.0KP sequence.

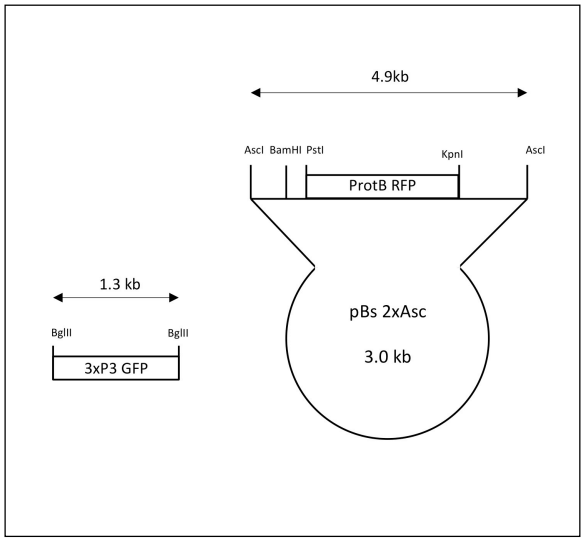
The 6.4 kb *Asc*I fragment of pBS/2xAsc/3xP3-GFP/ProtB-RFP was then cut out and ligated into the *Asc*I site of pBS/attB-FA to give pBS/attB/3xP3-GFP/ProtB-RFP (Fig. 10 c,d).

A 0.9 kb fragment containing the UAS-*rpr.c* cDNA sequence flanked by the UAS and the SV40 transcriptional terminator was PCR-amplified from transgenic flies of genotype w^{1118}*; P{*w^{+mc} *= UAS-rpr.C}14* (Bloomington Stock Center stock 5824), using primers pUAST 5' and pUAST 3. This product was then inserted into pCR4 using the TOPO TA cloning method. The insert was then cut out with *Fse*I and ligated into the *Fse*I site of pBS/attB/3xP3-GFP/ProtB-RFP (Fig. 11b). to give the final construct pBS/UAS-rpr.c/attB/3xP3-GFP/ProtB-RFP (referred to below as simply UAS-rpr.c GR) (Fig. 12a).

An additional UAS construct was also synthesized once it was determined that the UAS-rpr.C cytotoxic gene was ineffective (see below). This new construct contained the cytotoxic gene *UAS-PolyQ108* (Marsh, et al., 2000). For the construction of this plasmid, a sequence containing the 43Q-R-67Q repeats, or PolyQ108, between the upstream activation sequence and the SV40 transcriptional terminator was PCR-amplified from flies carrying a *UAS-PolyQ108* transgene (*w; pUAST Q108 #16*, kindly provided by Larry Marsh, University of California at Irvine) using primers 5.3pUAST polyQ and 3.2pUAST polyQ. The product was then inserted into pCR-4 TOPO via TOPO TA cloning. The subsequent UAS-PolyQ108 fragment was then cut out with *Fse*I and ligated into the *Fse*I site of pBS/attB/3xP3-GFP/ProtB-RFP

(Fig. 11a) to give the final construct pBS/UAS-polyQ108/attB/3xP3-GFP/ProtB-RFP (referred to below as UAS-polyQ GR) (Fig 12b).

The identity of UAS-polyQ GR and Pros25-GAL4-RG was then checked using PCR (Fig. 13)



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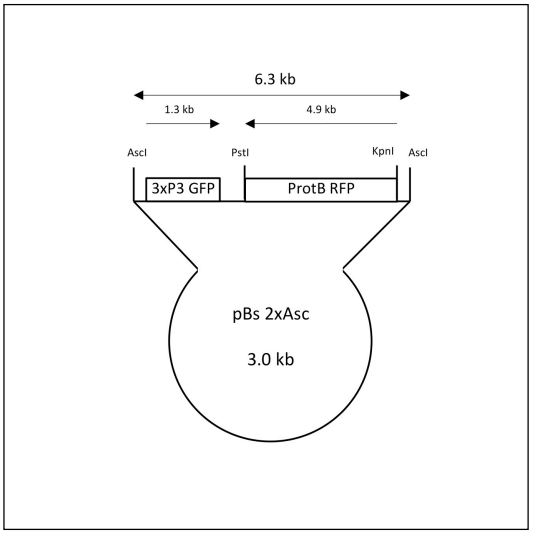

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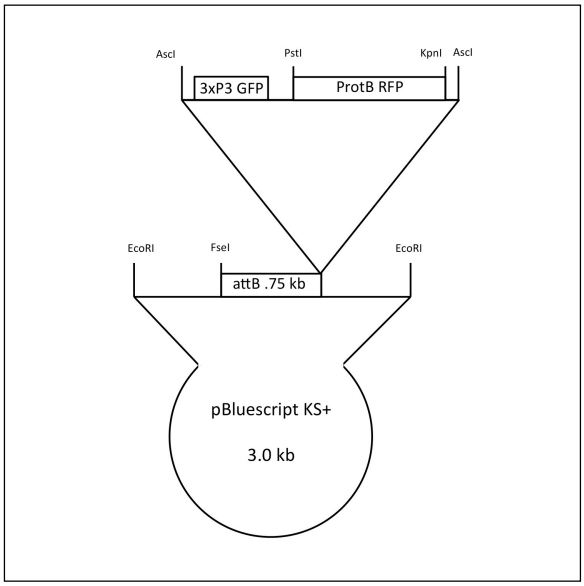

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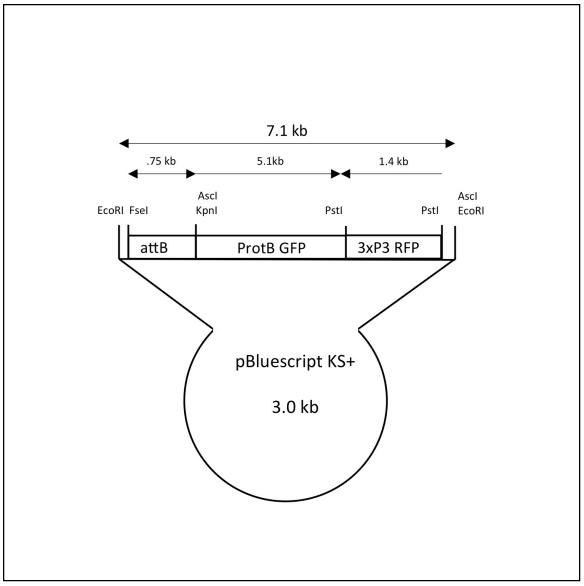
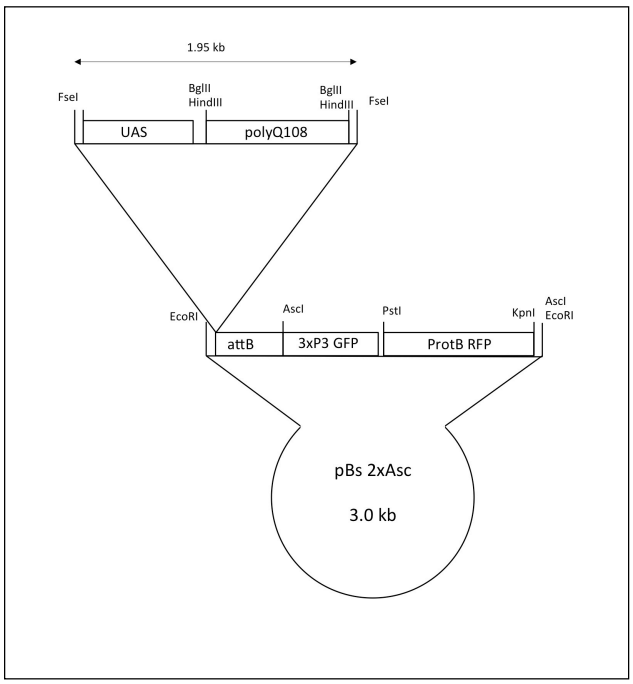


D

Figure 10 Construction of pBS/attB-FA/3xP3 GFP/ProtB-RFP. **A.** 3xP3 GFP fragment in pCR-2.1 TOPO is ligated into pBS/2xAsc/ProtB RFP **B**. The obtained pBS/2xAsc/3xP3 GFP/ProtB RFP construct **C**. Insertion of 3xP3 GFP/ ProtB RFP AscI-AscI fragment into pBS/attB-FA. **D.** Final pBS/attB/3xP3 GFP/ProtB RFP construct



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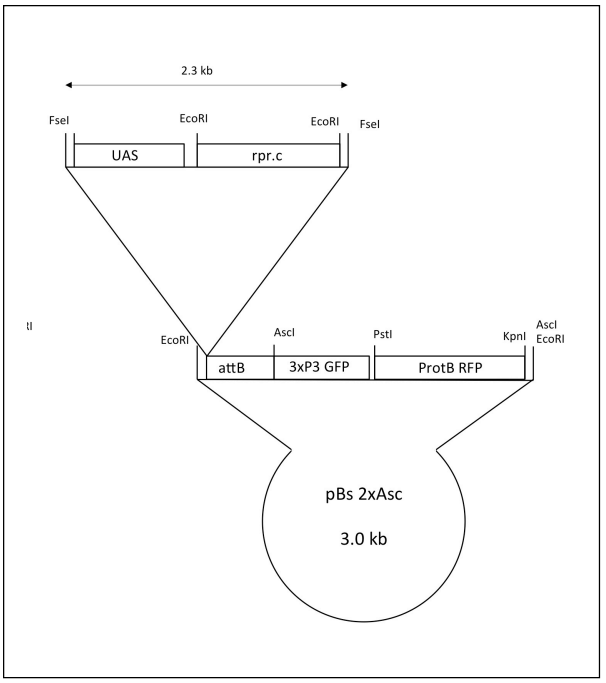

B

Figure 11 Insertion of UAS-polyQ108 and UAS-rpr.c into pBS/attB/3xP3 GFP/ProtB RFP

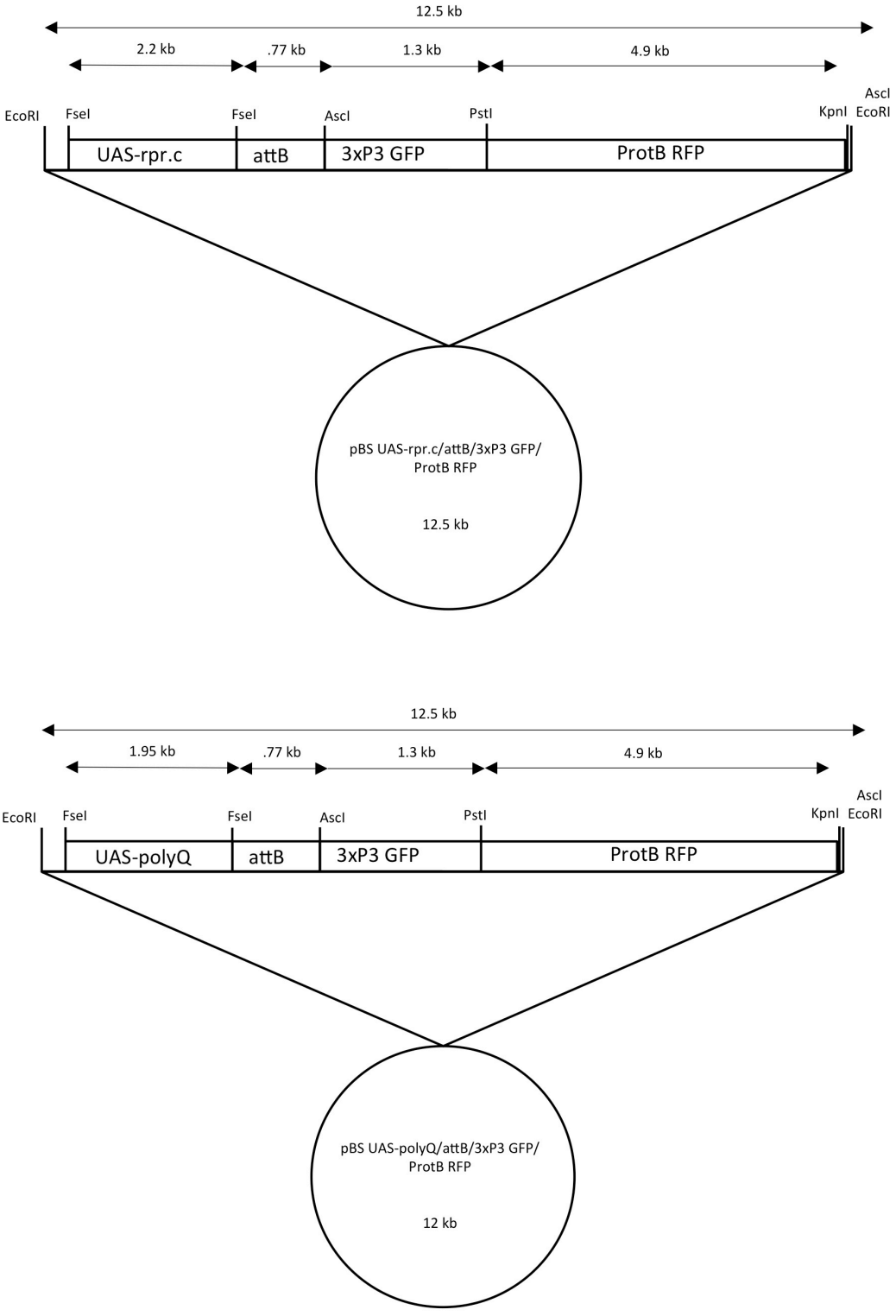

Figure 12 Final constructs. pBS/UAS-rpr.c/attb/3xP3 GFP/ProtB RFP and pBS/UAS-polyQ108/attb/3xP3 GFP/ProtB RFP

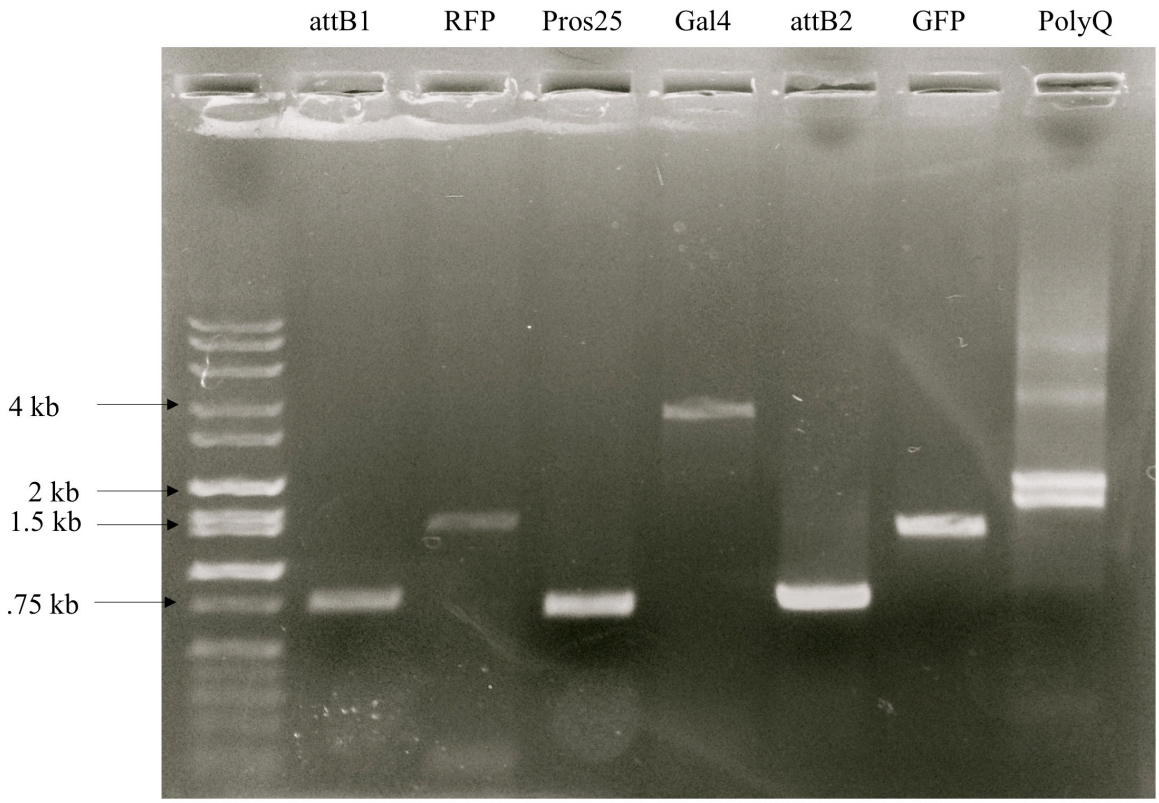

Figure 13 Results of the PCR confirming the components of constructs, UAS-polyQ108 GR and Pros25-GAL4 RG

Introduction of gene complex constructs into the genome using the ϕC31 targeted integration system

Transgenic constructs can be introduced into predetermined sites in the *D. melanogaster* genome using the ϕC31 integration system along with the recombinase-mediated cassette exchange (Groth et al., 2004; Venken, et al., 2006; Bischof, et al., 2007). Initially, two different lines were used as recipient hosts: *y[1]M{vas-int.Dm}ZH-2A w [*]; PBac{y[+]-attP-3B}VK00001* containing a ϕC31 integrase gene driven by a germline promoter on the X and a ϕC31 integrase *attP* target site on chromosome 2 at 59D, and *y[1]w[*]P{y[+t7.7]=nos-phiC31\int.NLS}X; PBac{y[+]-attB-9A}VK00027* containing a ϕC31 integrase gene driven by a germline promoter on the X and a ϕC31 integrase *attP* target site on chromosome 3 at 98E (Bloomington Stock Center stocks 24861 and 35569, respectively). In this system, the integrase enzyme catalyzes the insertion of the injected plasmid DNA, containing an *attB* sequence, into a genomic *attP* sequence that has been introduced via P-element or piggybac transformation. By using the same recipient *attP* line, one can reproducibly insert transgenes into the same genomic site. I chose as recipients lines that have the *attP* sequence located in the genome at a site that is relatively far removed from nearby genes, so that position effects on the inserted transgenes would be minimized.

Plasmid DNA (UAS-polyQ GR, UAS-rpr.c GR, or Pros25-GAL4) was ethanol precipitated and resuspended in injection buffer. Pre-blastoderm embryos were injected and the surviving larvae were transferred into vials and

the resulting adults single-pair mated with wild-type (wt) LH_m mates. Progeny were scored and transformants identified by their fluorescent eye phenotype.

Because the Chromosome 2 host strain (: *y[1]M{vas-int.Dm}ZH-2A w [*]; PBac{y[+]-attP-3B}*VK00001) had eye-specific GFP and RFP marker genes on the X (associated with the ϕC31 integrase transgene), we were limited to scoring sons from crosses set up with injected male parents crossed to wt females, since they do not inherit the double-marked X. For the Chromosome 3 host (*y[1]w[*]P{y[+t7.7]=nos-phiC31\int.NLS}X; PBac{y[+]-attB-9A}VK00027*), both males and female parents could be used, and all progeny could be scored for fluorescent eyes. Since the first transformant discovered was found to be on chromosome three, subsequent injections were made using only this strain.

The generation of transgenic lines was successful for the Pros25-GAL4RG and UAS-rpr.c GR constructs. The generation of flies carrying the UAS-polyQ GR construct is still ongoing, and therefore no results can be presented here.

Characterization of a transgenic line carrying Pros25-GAL4RG

The Pros25-GAL4RG flies and the UAS-rpr.c GR transgenic flies were initially identified based on the eye-specific expression of the *3xP3-RFP* or *3xP3-GFP* gene, respectively, using a fluorescent microscope (see Fig. 14 for demonstration of this). Males from these transgenic lines were then dissected and their testes examined for the sperm head-specific expression of

the *ProtB-GFP* or *ProtB-RFP* genes. In addition, females from the homozygous Pros25-GAL4RG flies and the UAS-rpr.c GR strains were dissected, after mating with males of the same strain, and their sperm storage organs (seminal receptacle and spermathecae) were examined for fluorescent sperm (Fig. 15) As shown in the figure, the sperm are easily visible, and the differential fluorescence of the two lines demonstrates that it will be possible to carry out future studies looking at PMPZ mechanisms of sexual selection. Once the above expected expression patterns were confirmed, these two lines were backcrossed with *LHm* mates and then fixed to establish stable homozygous lines.

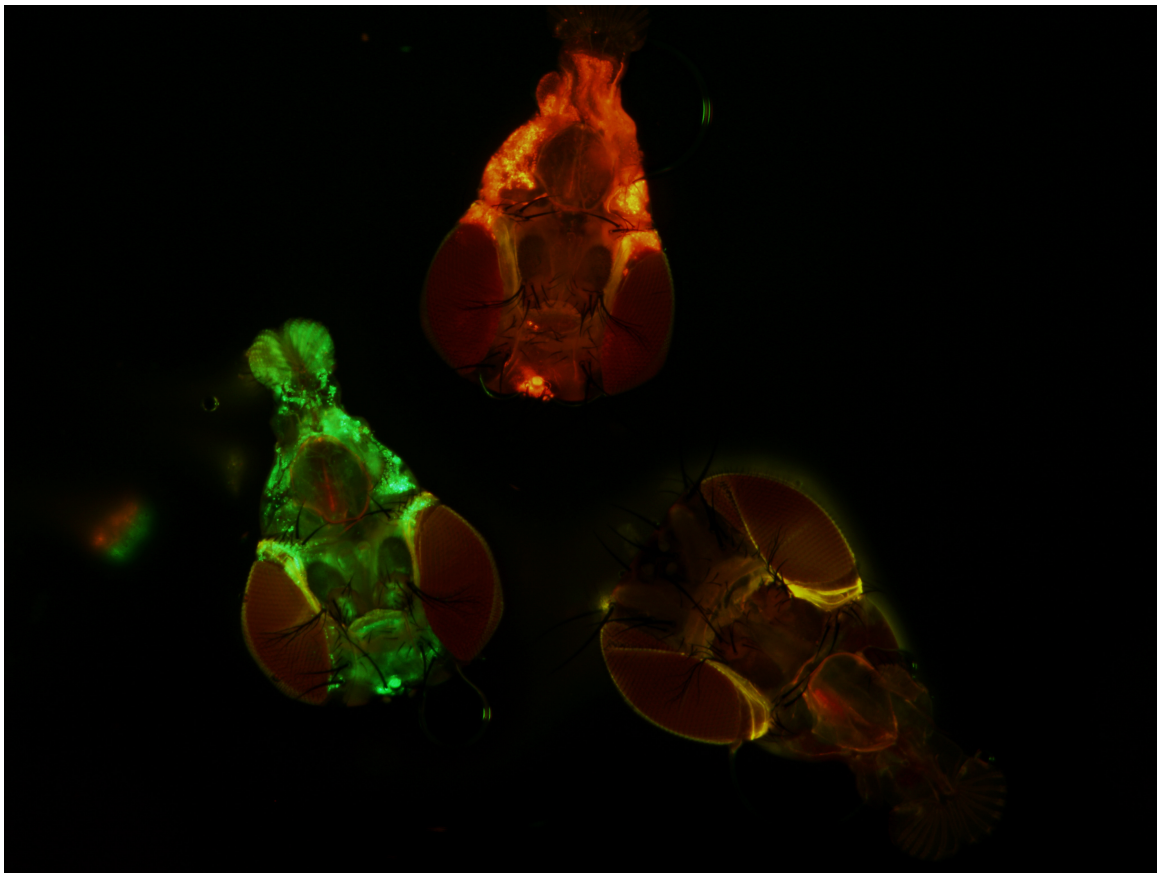

Figure 14. Fluorescent expression of 3xP3 GFP and RFP proteins in *Drosophila* heads. Clockwise, from bottom left: UAS-rpr.c GR, Pros25-GAL4 RG, *LHm* fly

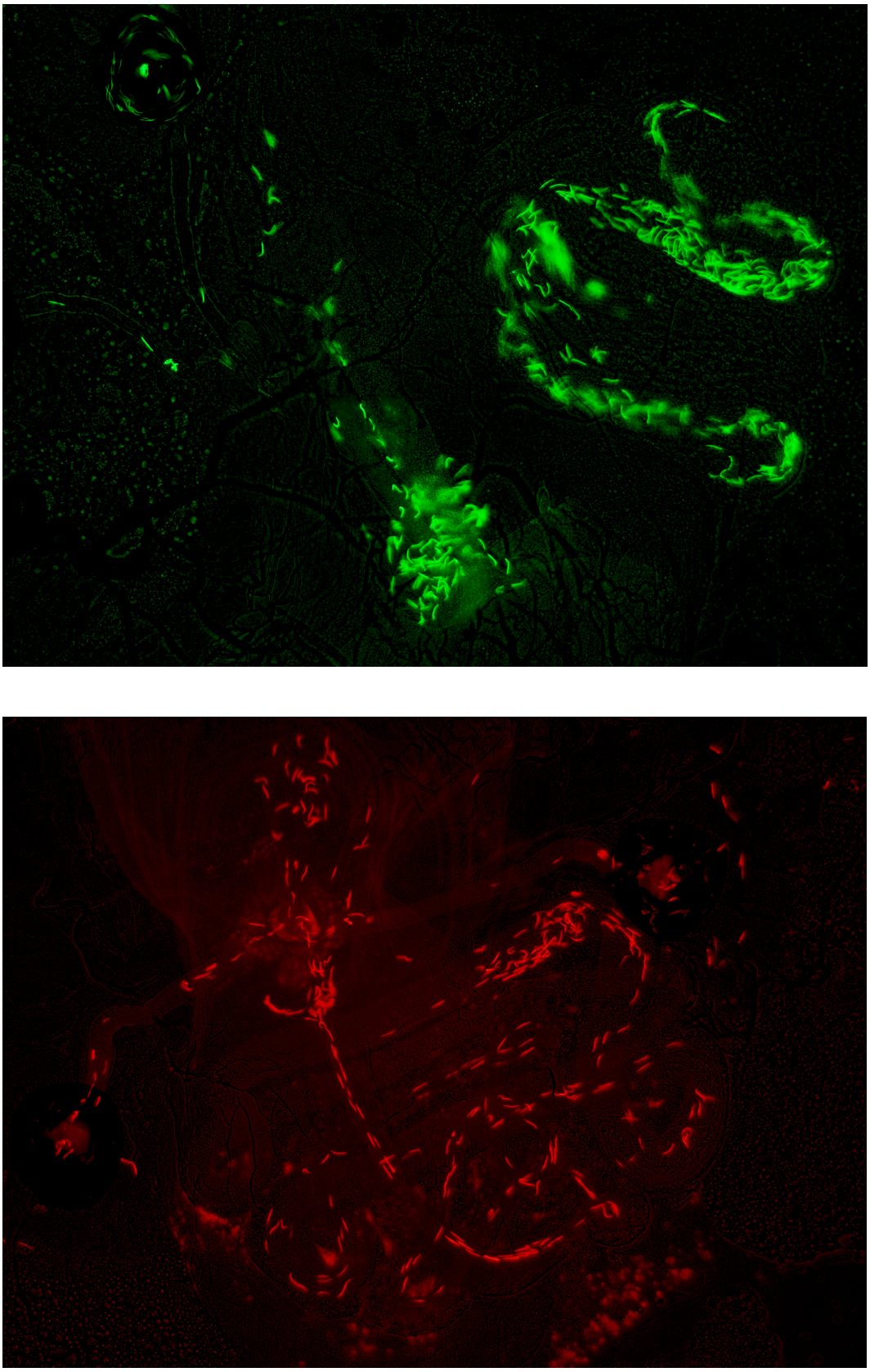

Figure 15 Green and red- labeled sperm heads of Pros25-GAL4 RG and UAS-polyQ108, under a fluorescent microscope. 100x.

Having established stable lines of both Pros25-GAL4RG and the UAS-rpr.c GR transgenics, I carried out a series of test crosses to assess whether these two lines had the expected properties. For example, the Pros25-GAL4RG line (the *A* allele) was crossed to a test strain that had GFP under the control of a UAS promoter (Bloomington Stock Center stock #4775, w^{1118}; $P\{w^{+mC} = UAS\text{-}GFP.nls\}14$). It was expected that the Pros25-GAL4 gene would drive constitutive expression of the UAS-GFP. Although GFP expression was observed in many tissues, the levels were relatively low, and some tissues did not exhibit any GFP expression. So, while it was clear that the *Pros25-GAL4* gene was working (*i. e.*, it was activating a UAS promoter) its expression level was not as great as anticipated.

To see if the UAS-rpr.c GR construct (i.e., the *B* allele) could induce programmed cell death when paired with a GAL4 driver, I crossed heterozygous UAS-rpr.c GR flies with flies of genotype w^{1118}; *P{GMR-GAL4. W*^{1}*2/CyO* (Bloomington Stock Center # 9146). This carries a driver that expresses GAL4 under the control of an eye-specific promoter. Thus, it was expected that the offspring that carry both UAS-rpr.c GR and GMR-GAL4 would show reduced eyes due to reaper induced apoptosis. As shown in Fig.16, this was seen, suggesting that the UAS-rpr.c GR transgene would be useful. However, on unanticipated problem was that many flies of the UAS-rpr.c GR line that were homozygous (i.e., *BB* flies) displayed a defective wing phenotype (curled and/or blistered wings, Fig. 17). This is unfortunate

because for the experimental evolution study that is proposed, it is important that the starting populations (*AA* and *BB*) have few if any fitness defects. Even more of a problem was seen when I crossed the UAS-rpr.c GR flies to the Pros25-GAL4RG transgenic line. Surprisingly, there was no lethality observed in the hybrid offspring.

Figure 16 Cross of UAS-rpr.c GR with GMR-GAL4 flies (two flies on the bottom) as compared to a wild-type *Drosophila* (top)

A

B

Figure 17 Self-expression of homozygous UAS-rpr.c GR. **A**. An *LHm* female Drosophila **B.** Homozygous UAS-rpr.c GR male fly.

Although the UAS-polyQ GR construct has yet to be introduced into the genome, there is a strain of flies that carries the *UAS-PolyQ108* construct, *w; pUAST Q108 #16* (Marsh, et al., 2000). Therefore, I was able to test whether the *Pros25-GAL4* driver would trigger lethality in hybrid offspring carrying both Pros25-GAL4 RG and the *UAS-PolyQ108* gene. For this experiment, crosses were set up with Pros25-GAL4RG males crossed to *w; pUAST Q108 #16* females and Pros25-GAL4RG females crossed to *w; pUAST Q108 #16* males. As a negative control Pros25-GAL4RG females were crossed to wild-type males of the *LHm* strain. As seen in Table 1, there was 100% lethality in the *Pros25-GAL4/UAS-PolyQ108* hybrids, with the strongest lethal phase during pupal development. This result is very encouraging, as it suggests that the UAS-polyQ GR transgenic line, once it has been generated, will be genetically incompatible with the Pros25-GAL4RG line.

	Pros25-GAL4 RG m x UAS-polyQ 108 f	**Pros25-GAL4 RG f x UAS-polyQ 108 m**	**Pros25-GAL RG f x *LHm* m**
eggs	128	137	83
hatched	83	91	81
pupae	73	81	72
viable adults	0	0	72

Table 1. Crosses of Pros25-GAL4 RG with UAS-polyQ show 100% lethality as compared to 13% lethality in Pros25-GAL4 RG and *LHm*

Future work

The results described above indicate that the experimental strategy for generating engineered populations of genetically incompatible *D. melanogaster*, carrying dominant genetic markers, and differentially tagged sperm heads, is feasible. I have successfully constructed two plasmids with multi-component gene complexes that have the desired properties. That is, the *A* construct has a 3xP3-RFP gene that effectively labels the eyes fluorescent red, and a ProtB-GFP gene that labels the sperm heads with green fluorescence. In addition, the attB sequence has been shown to target the plasmid vector to the genomic attP site using the ϕC31 integrase transformation system. Finally, it carries the *Pros25-GAL4* gene which can trigger pupal lethality when paired with the *UAS-PolyQ108* gene. Similarly, the *B* construct has a functional 3xP3-GFP gene that results in green fluorescent eyes, and a ProtB-RFP gene that produces red fluorescent sperm heads. Successful transformation of the UAS-rpr.c GR construct shows that the attB sequence is functional for targeted integration. While its disappointing that the UAS-rpr.c GR construct did not trigger hybrid lethality when paired with Pros25-GAL4RG, the results described above strongly suggest that the UAS-polyQ GR transgenic line, once it has obtained, will be effective at causing hybrid lethality when crossed with Pros25-GAL4RG.

Once the UAS-polyQ GR transgenic line is obtained, it will be backcrossed with wild-type for several generations and then fixed. While it is beyond the scope of my Capstone Project, future work will involve using

these engineered *A* and *B* populations to carry out a long term laboratory evolution study, examining the rapid evolution of additional reproductive barriers, and dissecting the various isolating mechanisms (either pre-mating or PMPZ) that can appear during the course of reinforcement.

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* Figures of both pCR2.1-TOPO and pCR4-TOPO were taken from invitrogen.com website

CAPSTONE SUMMARY

Speciation occurs when two populations of the same species can no longer reproduce, either because of pre-mating reproductive isolation, which includes habitat, temporal, or behavioral isolation, or due to post-mating pre-zygotic (PMPZ) isolation, where mechanical or gametic isolation renders the strains incompatible. Previous work by Daniel Matute (Univ. of Chicago) has shown that when incipient species begin to experience reproductive isolation, a phenomenon called "reinforcement" can accelerate the process of speciation, by the rapid evolution of new pre-mating or PMPZ barriers. While the occurrence of reinforcement has been studied for many years, not much is known about how rapidly natural selection can create new reproductive barriers, or what the actual mechanisms are that are likely to arise (e.g., pre-mating mechanisms like changes in courtship behavior, or PMPZ mechanisms like differential sperm storage or use by the female).

In my Capstone project, under the supervision of Dr. John Belote and in collaboration with the Scott Pitnick lab, I aimed to develop an experimental approach to observe the process of reinforcement in a laboratory setting, and to examine in detail the types of pre-mating and/or PMPZ mechanisms that can rapidly evolve following an experimentally-induced hybrid incompatibility.

Specifically, my project focuses on using genetic engineering to create populations of genetically incompatible *Drosophila* strains, which then will be studied to understand how strains might diverge from each other after genetically inducing hybrid inviability. For example, after mating, female *Drosophila* stores sperm in two storage organs: the seminal receptacle and spermathecae. If she mates with another male, the female usually displaces sperm of the first male and uses the newer sperm for fertilization. However, if she mates first with a male of her same strain, and then mates with a genetically incompatible male, it is possible that this pattern will shift, and that the number of first male's progeny will be higher than that of the second male's. This phenomenon, known as "cryptic female choice", is an example of one possible mechanism of a reproductive barrier that might arise following initial genetic incompatibility, demonstrating the phenomenon of reinforcement. This type of study has relevance for understanding biological diversity and *D. melanogaster* mating preferences.

I specifically focused on engineering populations of genetically incompatible *D. melanogaster*. The first step was to use recombinant DNA methods to create plasmid constructs that when introduced into the fly genome would act as incompatible "alleles" of a single locus. Flies homozygous for either allele (called A and B) are viable and fertile, while heterozygous AB hybrids are be lethal. Populations of the A and B strains were then placed in large cages where they were allowed to interbreed for many generations. Because of the hybrid incompatibility, the only productive

fertilizations involve gametes of the same strain (A x A or B x B). It is predicted that this will drive the rapid evolution of pre-mating or PMPZ mechanisms favoring such mating, and selecting against the occurrence of A x B matings. This experimental system is designed such that these newly evolved mechanisms can be recognized and studied in detail. For example, for studying pre-mating events, individuals of strain A and B will be easily recognized by their red or green fluorescent eye spots, while PMPZ mechanisms can be studied by virtue of the A and B stored sperm being distinguished by their green or red fluorescent sperm heads.

Figure 1 gives a schematic representation of the A and B synthetic alleles of *D. melanogaster* that I have created. These gene constructs consist of four components. (1) The Prot-B RFP and Prot-B GFP sequences encode sperm specific proteins tagged with red fluorescent proteins or green fluorescent protein for the clear distinction of A and B sperm within the female's seminal receptacle. (2) The 3xP3 RFP and 3xP3 GFP cassettes result in eye-specific expression of RFP and GFP to allow easy identification of which allele each fly carries. (3) The attB sequence allows these constructs to be inserted into a specific chromosomal site, using the phiC31 integrase system for site-specific transformation.(4) Finally, the engineered alleles contain one of the two components of the GAL4/UAS system for targeted gene expression. GAL4 is a yeast transcription factor that normally has no function in flies. In this construct GAL4 is expressed under the control of the *Pros25* promoter that normally drives constitutive expression of a proteasome gene. The *UAS*-

*polyQ*108 component has the toxicity inducing *polyQ108* sequence downstream of the GAL4 responsive regulatory sequence UAS. By itself, *UAS-polyQ108* has no effect, but in the presence of GAL4 (such as in AB hybrids) it induces programmed cell death, killing the embryo.

Engineering each of these constructs requires selecting for genes and various regulatory sequences and ligating them in such a way to ensure smooth insertion of the construct and its subsequent effectiveness in the flies. First, I designed the primers that would allow the amplification of all of the gene fragments using the Polymerase Chain Reaction (PCR). Then, I cloned the amplified fragment into the TOPO plasmid vector, and screen for the desired product using restriction enzyme digestions. I then confirmed the DNA of interest by gene sequencing. When found, I subsequently subcloned the gene fragments and ligated them together (for example, 3xP3 GFP gene fragment into the pBS/ 2xAsc/ProtB RFP plasmid) using gel extraction and ligation. The process is then repeated where every gene fragment would be added to the growing construct. Once the remaining constructs are completed, a midi-prep is done for isolating good quality DNA for the injection into *Drosophila* eggs for germline transformation.

Next, the plasmid constructs were introduced into the fly genome using the phiC31 integrase system for site specific transformation to ensure that the insertion of the construct in itself will not repress the normal gene expression of *Drosophila.* Once the injected flies developed, I crossed them to the wild-

type flies and screened the progeny for the constructs using a fluorescent microscope.